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*EFFECTS OF MECHANICAL SHEAR ON GENETIC ACTIVITY OF BACILLUS SUBTILIS DNA**

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DNA molecules in solution can be fragmented by mechanical shearing forces.[1] Such fragmentation disrupts the linkage of genetic markers arrayed on the DNA molecule of the bacteriophage lambda.[2] In *Bacillus subtilis*, the aromatic linkage group carries at least nine genes specifying structural enzymes for steps in aromatic acid biosynthesis and also a histidine marker.[3, 4] This situation affords an unusually favorable opportunity to relate the biological to the physical consequences of shearing forces of DNA in a transformation system.

Materials and Methods.—Pertinent information on the strains carrying the linked mutants is given in Table 1 and linkage map, Figure 1. All loci, except for aro_2 and aro_3, were ordered on

TABLE 1

LIST OF STRAINS OF *Bacillus subtilis*

Strain No.	Genotype[1]	Growth response
23	thr^-	threonine
168	try_2^-	tryptophan
SB19	Reference Prototroph	—
SB25	$his_2^-try_2^-$	histidine + tryptophan
SB70	$his_2^-tyr_1^-$	histidine + tyrosine
SB130	$his_2^-aro_1^-$	histidine + tyrosine + phenylalanine + tryptophan
SB137	$his_2^-aro_2^-$	histidine + shikimic acid
SB148	$his_2^-aro_3^-$	histidine + shikimic acid
SB202	$aro_2^-try_2^-his_2^-tyr_1^-$	shikimic acid + tryptophan + histidine + tyrosine
SB402	$aro_3^-his_2^-aro_1^-$	shikimic acid + histidine + tyrosine + phenylalanine + tryptophan

[1] The nomenclature of genotypes follows the suggestions of Demerec[16] and the abbreviations coincide with the designations given by the editors of the *Journal of Biological Chemistry*.

the basis of three-point crosses on the assumption that the least frequent class results from a quadruple crossover.[4] A two-point map distance (q) can be stated as the ratio of crossover to total transformants with respect to the markers tested.[5] The mapping of the loci by this procedure accords with the ordering based on the three-point tests.

Media—Difco Antibiotic medium 3 = A3; Davis mineral[6] = D. Competency regimen—CHT-1 and CHT-10 with aromatic supplements added.[4]

DNA was assayed at limiting levels: no more than 0.02 μg per ml of 5×10^8 recipient cells.

DNA was initially prepared as previously described.[7] In later experiments, it was prepared

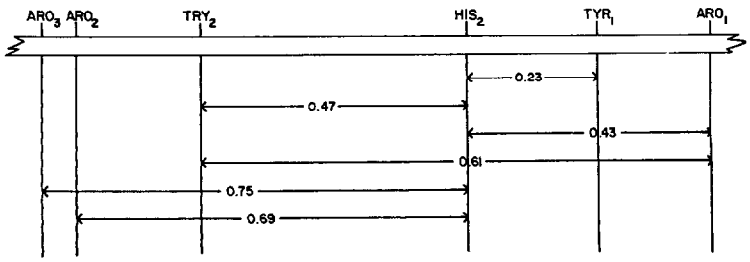

FIG. 1.—Linkage map of loci of aromatic amino acid biosynthesis in *Bacillus subtilis*. Map distance $q = (10 + 01)/(10 + 01 + 11)$.

according to the procedure of Marmur,[8] which includes a minimum of two precipitations of the final ethanol precipitate in 0.54 volumes of isopropanol. The location of the peaks of radioactivity and biological activity was the same for both preparations assayed by sucrose gradient sedimentation. In many preparations, the isopropanol precipitation removed a shoulder of P^{32} activity which was biologically inactive, trailing the major peak and mostly solubilized by RNase.

For the preparation of P^{32}-labeled DNA, bacteria were grown under the conditions described by Young and Spizizen.[9] In some preparations, two aliquots of unlabeled bacteria grown in A3 were added as a carrier immediately prior to the DNA isolation. The behavior of biological activity and P^{32} banding corresponded to preparations when no such carrier was added. The quantities of P^{32} employed resulted in a maximum of 0.6 atoms of P^{32} per 10^5 atoms of P^{31} DNA phosphorus, or 0.2 atoms per DNA molecule of molecular weight 10^7. Bihelix breakage during storage was not detected when samples of a single preparation were centrifuged periodically over four weeks, the longest a preparation was ever used. DNA concentration was routinely determined and expressed as optical absorbance at 260 $m\mu$.

Shearing: Solutions of DNA, with an absorbance of 0.5 in 0.14 *M* sodium chloride + 0.015 *M* sodium citrate, were stirred at 6,000 rpm with a Virtis Homogenizer micropropeller with crossed blades. A volume of 1.3 ml was used in a fluted micro-cup, having a total capacity of 2 ml. The cup was surrounded with an icebath. Varying the DNA concentration between 0.05 and 0.5 absorbance units, or the salt concentration between 0.14 *M* and 2.0 *M* NaCl, in 0.15 *M* sodium citrate did not appreciably affect the kinetics nor extent of biological inactivation.

Viscosity was measured in a single-bulb capillary viscosimeter at 37° employing DNA concentrations of 0.5 absorbance in 0.14 *M* NaCl + 0.015 sodium citrate. Flow time of the diluent was 102.0 sec.

Experimental Results.—Breakage of a DNA molecule may be evidenced biologically as the disruption of genetic linkage, i.e., the loss of multiple cotransfers relative to single transfers. A variety of shearing methods was evaluated by the ratio of single-marker transfer to linkage group cotransfer to SB 190, i.e., try_2 compared to aro_2-his_1: (1) Squirting the DNA solution under pressure through various-sized hypodermic needles,[10] (2) spraying from an atomizer,[11] (3) sonic oscillation,[12] and (4) stirring in a Virtis homogenizer.[2] Each of these techniques showed some discriminating effect for breaking the molecule, but the stirring technique was most effective in our hands and was used exclusively in these studies.

Figure 2 displays the results of a kinetic study of the transfer of the linkage group, aro_3-aro_1 (recipient SB 402) by wild type DNA after progressive shearing. After one min, there is a rapid and progressive decrease in singlet, doublet, and triplet transfers respectively. After 20 min, there is no further decrease in singlet transfer, whereas the activity for the doublets and triplets continues to drop. Like the biological activity, most of the viscosity increment has been reduced by 5 min. The further measurements of the approach to the asymptotic viscosity are too imprecise to justify further comparisons since a small fraction of undegraded DNA molecules would contribute disproportionately to the biological activity as compared with the viscosity.

Although the data in Figure 2 suggest bihelix breakage, the possibility that damage at random sites along the DNA molecule results in inactivation of only portions of the linkage group has not been rigorously excluded. In order to distinguish damage in an intact bihelix from breakage, the biological activity of DNA should be expressed as a function of the size of the DNA molecules.

The physical characteristics of sheared versus intact DNA (both labeled with P^{32}) were studied by sedimentation in a liquid column stabilized by a sucrose density gradient. Fractions were collected and assayed for radioactivity and for biological

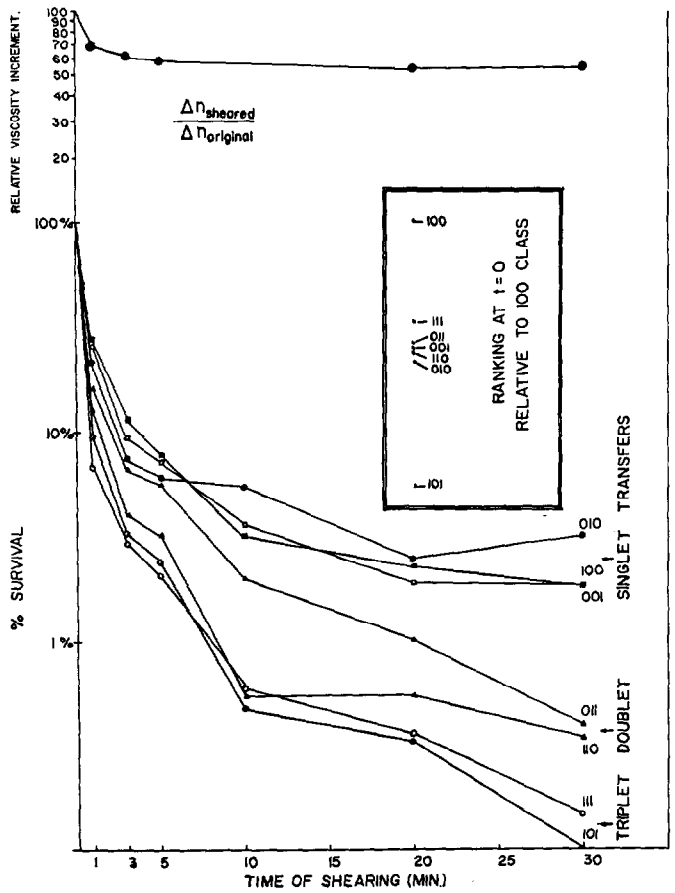

FIG. 2.—Kinetics of the effect of shearing on transforming activity and viscosity. 1.3 ml of SB 19 DNA, 0.5 O.D. units per ml, was sheared at 6,000 rpm for the indicated time. The total sample was assayed for viscosity and an aliquot diluted for the transfer of the linkage group aro_3-aro_1. The platings were done on doubly supplemented media, and in most cases a total of 600 representative colonies of all three primary transformant classes were analyzed for the remainder of their genotype by replicate plating onto appropriately supplemented media. However, because of the reduction in biological activity, 390, 297, and 266 colonies were analyzed for the time periods 10, 20, and 30 min respectively.

activity against the recipient strain SB 202 (aro_2^-, try_2^- his_2^- tyr_1^-). We ask: (1) Are all size classes of DNA equally active in transformation? (2) Does shearing change the sedimentation characteristics of the DNA?

Unsheared sample (Fig. 3): The biological activity of the DNA with respect to try_2^+ transfer varies with the sedimentation constant; the peak for P^{32} (total DNA) is at $S = 6.9$; the peak for biological activity is at $S = 10.0$.[13] This displacement has been observed in every DNA preparation studied, including DNA isolated from exponentially growing cells pulse-labeled for only 10 min, and DNA containing a large fraction of unlabeled carrier DNA.

Sedimentary fractionation of markers in the DNA was investigated by assaying these same fractions for transfer of the aro_2 marker (linked to try_2) as well as four markers unlinked to the try_2 locus and to each other: uracil, histidine, methionine,

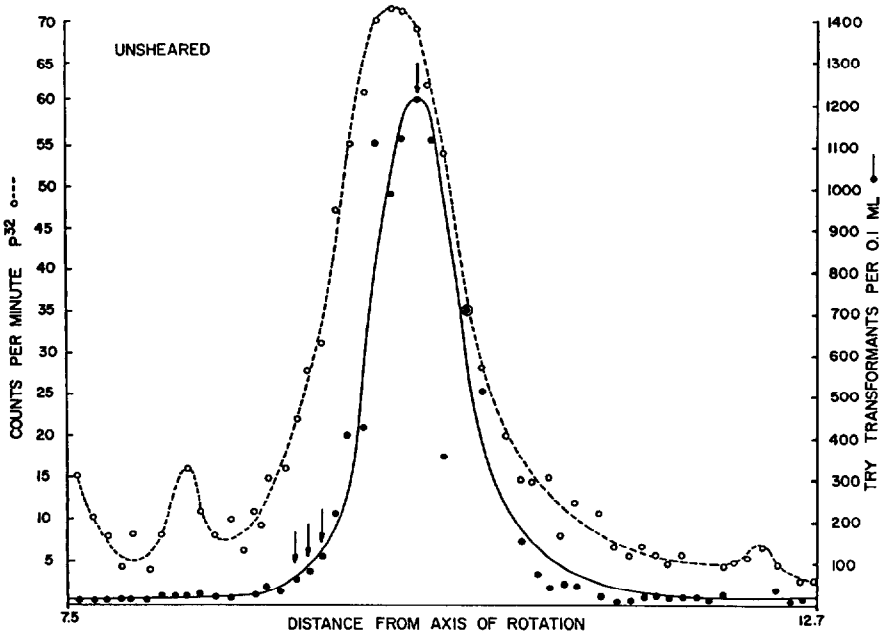

FIG. 3.—Sedimentation of unsheared DNA. 0.1 O.D. units of P^{32}-labeled DNA, prepared by the Marmur procedure, was layered on top of a sucrose gradient column of 24 ml, the sucrose concentration decreasing linearly from 20 per cent wt/vol at the bottom to 5 per cent at the top. The solution was centrifuged for 12 hours at 20,000 rpm at 6 per cent in the SW 25.1 rotor of the Spinco Model L centrifuge. The rotor was allowed to stop without braking, and 0.45-ml samples were collected dropwise through a hole punctured in the bottom of the centrifuge tube. 0.1 ml aliquots were added to 0.9 ml of competent bacteria and transformants assayed as previously described. 0.2 ml were plated on planchets and assayed for radioactivity. The fractions may be numbered, in the order in which they are collected, from right to left. The arrows point to fraction numbers 31 and 38–40.

and phenylalanine. All of these markers sedimented together, indicating that the biologically active DNA comprises only a portion of the total DNA isolated, namely, the larger molecules. The biologically inactive DNA may consist of partially degraded material.

Fractions 30, 31, 32, the tubes of peak biological activity, as well as fractions 38, 39, 40, corresponding to the peak of activity of sheared DNA, were assayed for the cotransfer of aro_2-tyr_1 (recipient strain SB 202). The transfer of this segment of the linkage group is essentially the same in all of these fractions (Table 2) from this preparation.

TABLE 2

COTRANSFER OF aro_2-tyr_1 MARKERS BY UNSHEARED DNA

Fraction No.[1]	No. transformants scored[2]	Try_2^+ Transformant Genotypes (per cent of total transformants)							
		1111[3]	1110	1101	0111	1100	0110	0101	0100
30	200	19	2.5	1	19.5	27	6	1.5	23.5
31	200	21.5	5	1	21.5	22	6	1.5	21.5
32	200	32	6.5	0	17.5	25	3.5	0	16.5
38	100	17	5	1	21	24	4	1	27
39	80	18.7	3.8	0	21.3	28.8	5	3.8	18.7
40	60	23.3	3.3	1.6	15	26.5	1.6	1.6	28.3

[1] Fraction No. refers to designation in Figure 2.
[2] The indicated number of try^+ transformants were stroked onto nutrient agar plates with sterile toothpicks (50 per plate). These plates were then replicated to appropriately supplemented media to determine the remainder of the genotype.
[3] The donor markers are designated as 1 and the recipient markers 0.

The unsheared material has also been run through the methylated albumen column described by Mandell and Hershey.[14] In contrast to the results from the previous sedimentation experiments (involving different DNA preparations), some fractionation of molecules according to their capacity for quadruplet versus singlet transfer is indicated, with the genetically smaller fragments eluting at the lower salt concentrations, suggesting their decreased physical size. In concordance with the previous sedimentation results, the peak of biological activity is displaced from the peak of DNA concentration. Again, the genetically longer fragments possess the higher biological activity.

After shearing, the sedimentation diagram shows P^{32} activity at $S = 5.7$ (Fig. 4). No more than a few per cent, at most, of unsheared material at $S = 7.1$ can be seen. Again, the biological activity is biased towards the high S side of the peak of P^{32} activity ($S = 6.5$). To test whether sheared DNA molecules are genetically cleaved, try_2 transformants were scored for the remainder of their genotype (Table 3). There is a marked reduction in the proportionate cotransfer with try_2 of linked markers in the sheared compared with unsheared material. The quadruplet (1111) and triplet (1110) transfers show the most striking responses.

Correlation of map distance and shear effectivity: Shear forces should cleave distant loci while leaving closer genetic loci still intact. Wild type DNA, sheared for 30 min, was assayed for cotransfer of his_2 and a series of markers located at various map distances from it. The furthest markers are more readily separated (Table 4). Different DNA samples often show different absolute values for r/r_s, but the correlation remains.

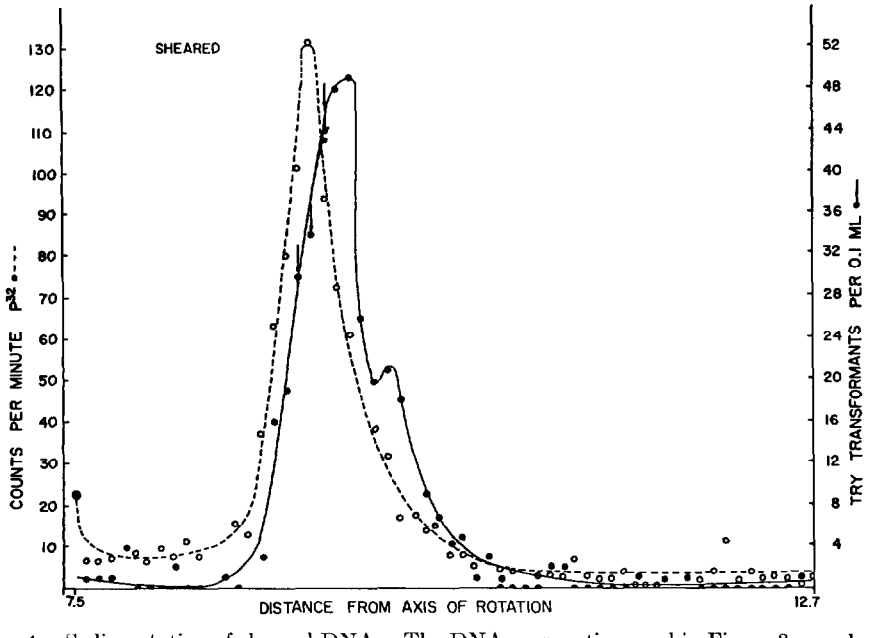

FIG. 4.—Sedimentation of sheared DNA. The DNA preparation used in Figure 3 was sheared for 30 min at 6,000 rpm, resulting in 97 per cent loss in transforming activity of the try_2 marker. 0.05 O.D. units were layered on a sucrose-gradient column, and the remainder of the centrifugation, collection, and assay were done as described under Figure 3. The arrows indicate fractions 40–42.

TABLE 3

COTRANSFER OF aro_2-tyr_1 MARKERS BY SHEARED DNA

Fraction No.[1]	No. transformants scored[2]	Try_2^+ Transformant Genotypes (per cent of total transformants) 1111	1110	1101	0111	1100	0110	0101	0100
40	195	2.4	2.6	0	7.2	23.6	10.2	0.5	53
41	175	2.3	2.8	1.1	6.3	22.3	9.8	2.3	53
42	128	0.8	0.85	0	13.2	18.0	6.2	1.56	44

[1] Fraction No. refers to the designation in Figure 4.

[2] The indicated number of try^+ transformants were stroked onto nutrient agar plates with sterile toothpicks(50 per plate). These plates were then replicated to appropriately supplemented media to determine the remainder of the genotype.

TABLE 4

CORRELATION OF COTRANSFER INDEX OF his_2 LINKAGE WITH VARIOUS MARKERS BEFORE AND AFTER SHEARING

Markers	Cotransfer index before shearing, r	Cotransfer index after shearing, r_s	Relative effect of shearing, r/r_s
his_2-tyr_1	0.77	0.23	3.3
his_2-aro_1	0.57	0.12	4.2
his_2-try_2	0.53	0.10	5.3
his_2-aro_2	0.31	0.05	6.2
his_2-aro_3	0.25	0.015	16.7

The DNA preparation was assayed on the indicated double auxotrophs before and after shearing for 30 min at 6,000 rpm. The cotransfer index was calculated from the transformant counts on appropriately supplemented media.

Discussion.—The present observations on the loss of biological activity of DNA degraded by shear forces extend similar work done on pneumococcal DNA after shear degradation. We can also correlate a physical breakage of the DNA bihelix with the disruption of linkage of genetic loci located in nucleotide sequences on either side of the point of breakage.

In the present studies, the DNA extracted from *B. subtilis* has a sedimentation constant of 26.7 measured at 20°C using a DNA concentration of 25 μg per ml and an "Epon" centerpiece in the Spinco Model E analytical ultracentrifuge. This value compares favorably with the sedimentation constant of 30 obtained by Burgi and Hershey[15] for a sample of *E. coli* DNA. This size corresponds to one-quarter molecules of the T2 DNA of molecular weight 120×10^6. In contrast to the phage DNA, the molecules of *B. subtilis* DNA are not of uniform length. Both P^{32} and biological activities yield a disperse band after velocity sedimentation and fractionate into different genetic sizes on the methylated albumin column. A variety of shear forces during DNA isolation have resulted in a product which may represent only a fraction of its molecular weight inside the cell.

The studies of the breakage of T2 and lambda DNA have led to the conclusion that at a critical shear rate the DNA molecule tends to break at a point near the middle of the molecule, resulting in two half-molecules not subject to further degradation at the same shear rate. In the present studies, because of the relatively low molecular weight of the DNA, the critical rate of shear was determined arbitrarily as that rate of shear required to give a distinction in the inactivation of transfer of a single, compared to two, linked markers. At the speeds of stirring necessary to give these rates of shear, the vortex dips into the DNA solution and results in the production of air bubbles and turbulence. The significance of such forces on the phenomena observed is not apparent.

Several observations suggest that the present results may likewise be interpreted as the transverse breakage of DNA bihelices to about half their original size:

1. The displacement of the sedimentation constant for peak biological activity is 0.65 (uncorrected for the gradient of the sucrose), corresponding to a ratio of 0.4 in molecular weight.[12]

2. The shearing reduces the specific viscosity of the DNA by forty per cent.

3. The shearing of DNA resulted in the production of a new population of molecules, more uniform than the original population, indicating a preferential cleavage of the larger molecules.

4. The critical rate of shear can vary within limits—7,500 rpm for 5 min resulted in a DNA preparation equivalent to 6,000 rpm for 30 min in its sedimentation characteristics.

Taken in concert, these data suggest that the molecules of DNA are being broken near the middle.

The kinetics of inactivation does not permit one to distinguish between the disruption of linkage from the separate losses of component markers. In fact, since there is a survivorship of two to three per cent for the single markers at 30 min, the survivorship expected for triplet transfer would be only 10^{-5} if the markers were subject to independent loss. The actual survivorship of 111 is one hundred times higher than this. Therefore, the loss of single markers from triplet transfers cannot occur independently. This would be true if, for example, the breakage of a molecule tended to leave some markers intact in a smaller fragment, which was then relatively insensitive to further shearing. This view would also be consistent with the time course of inactivation and of loss of viscosity.

The study of shearing effects points up a number of problems covering the heterogeneity of "untreated" DNA. This has been studied by two independent techniques: velocity sedimentation and fractionation on a methylated albumin column. Both techniques indicate that the DNA fractions of highest molecular weight are the most likely to become integrated into the recipient genome. However, both techniques might confound complexing with, say, protein with molecular-weight variation.

The heterogeneity of the DNA as now isolated poses delicate problems for rigorous interpretation of the shearing effects. The most plausible model is the disruption of multiplet transforming molecules into smaller fragments having singlet activity. However, the extent of marker inactivation and the frequency of singlet transfer with the initial material make it difficult to prove the essential point that a multiplet has been converted to a singlet unit. Instead, we might argue a selective inactivation of different types of units. This problem is mitigated in the lambda phage system by the initial higher incidence of cotransfer that can be demonstrated in phage infection.

Summary.—DNA, extracted from *Bacillus subtilis* by lysozyme lysis and purified by shaking with chloroform-octanol and ribonuclease treatment, is heterogeneous in its physical and genetic size. The larger fragments appear to have the greatest biological activity. The DNA can be sheared mechanically, resulting in a class of molecules possessing a lower sedimentation value, reduced viscosity, and a decreased genetic complement. Their physical properties suggest that the sheared DNA molecules are one-half their original size.

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